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Identification of a novel antibacterial compound 1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol active against persisters of *Pseudomonas aeruginosa*

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Abstract

Antibiotics typically fail to completely eradicate a bacterial population, leaving a small fraction of transiently antibiotic-tolerant persister cells intact. Persisters are therefore seen as a major cause of treatment failure and greatly contribute to the recalcitrant nature of chronic infections. The current report is focused on *Pseudomonas aeruginosa*, a Gram-negative pathogen belonging to the notorious ESKAPE group and, due to an increasing resistance against most conventional antibiotics, posing a serious threat to human health. Greatly contributing to the difficult treatment of *P. aeruginosa* infections is the presence of persister cells and elimination of these cells would therefore significantly improve patient outcome. In this study, a small-molecule library was screened for compounds that, in combination with the fluoroquinolone antibiotic ofloxacin, reduced the number of *P. aeruginosa* persisters as compared to treatment with the antibiotic alone. Based on early structure-activity relationship, 1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol (SPI009) was selected for further characterization. Combination of SPI009 with mechanistically distinct classes of antibiotics reduced the number of persisters up to 10^6-fold in both lab strains and clinical isolates of *P. aeruginosa.* Further characterization of the compound revealed a direct and efficient killing of persister cells. SPI009 caused no erythrocyte damage and demonstrated minor cytotoxicity. In conclusion, we identified a novel anti-persister compound active against *P. aeruginosa* with promising applications for the design of novel, case-specific combination therapies in the fight against chronic infections.

Introduction

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen best known as the dominant cause of life-threatening chronic airway infections in cystic fibrosis (CF) patients (1). Infections are also commonly found in highly vulnerable patients such as burn wound victims, immunocompromised individuals and patients residing in intensive care units (2, 3). In addition to an intrinsic resistance towards a wide variety of antibiotics, *P. aeruginosa* has the remarkable ability to acquire additional resistance mechanisms. Rapidly increasing resistance to multiple antibiotic classes, including the so-called last-resort polymyxins, classifies the pathogen as multidrug- or even pan-resistant and have caused *P. aeruginosa* infections to become increasingly difficult to treat (2, 4, 5). *P. aeruginosa* is 4th most common cause of healthcare-associated infections in Europe (8.9%) with 14% of all isolates reported as being resistant to at least three antimicrobial classes (6, 7). The World Health Organization (WHO) has recently ranked *P. aeruginosa* as 'critical' in the 'Global priority list of antibiotic-resistant bacteria to guide research, discovery and development of new antibiotics', emphasizing its clear clinical importance and encouraging the development of novel antibacterial therapies (8, 9).

Some infections however, prove difficult to eradicate despite the absence of clinically detectable resistance against the used antibiotic (10). This can be explained by the presence of persister cells, a small fraction of phenotypical variants in a genetically homogeneous population that is tolerant to treatment with high doses of antibiotics (11, 12). It is generally assumed that, upon successful treatment with a bactericidal antibiotic, the host immune system is able to cope with this small fraction of surviving cells. However, in situations where the immune system is compromised or bacteria are able to evade the immune system, persisters

63 become a threat to human health and are considered a major culprit in chronic infections (13).
64 Persister cells greatly contribute to the observed antibiotic tolerance in biofilms, are responsible
65 for the recalcitrant nature of chronic infections (10, 14, 15) and recently, evidence is gathering
66 that persister cells can facilitate the emergence of genetic resistance (16–19). Targeting
67 persisters is therefore likely to greatly improve patient outcomes in chronic infectious diseases.
68 Despite the clear clinical relevance of persisters, there are still few anti-persister compounds
69 described in literature (20). Contributing to this is the still limited knowledge on the
70 mechanisms behind persister formation in *P. aeruginosa*, the fact that multiple, redundant
71 pathways seem to be involved and the observation that the processes implicated in persistence
72 seem to be species-specific (21, 22). Therefore, the rational design of target-based anti-persister
73 therapies remains difficult.
74 Here, we describe the screening of a diverse set of small-molecule compounds that resulted in
75 the identification of the potent anti-persister compound SPI009. This molecule strongly reduces
76 or even completely eradicates persisters of *P. aeruginosa* when administered in combination
77 with mechanistically distinct antibiotics. Importantly, SPI009 retains its activity against several
78 clinical isolates, directly kills persister cells in a highly efficient manner and causes no hemolytic
79 effects.

Materials and methods

Bacterial strains, human cell lines and culture conditions. Bacterial strains were cultured in 1:20 diluted Trypticase Soy Broth (1:20 TSB) at 37 °C, shaking at 200 rpm. For solid medium, TSB was supplemented with 1.5% agar. Human HEK293-T cells were cultivated in DMEM medium containing 5% heat-inactivated FCS and kept at 37°C with 5% CO_2. The following antibiotics were used: ofloxacin, ceftazidime (Sigma Aldrich) and amikacin (Acros). Concentrations are indicated throughout the text. The small-molecule library was provided by the Centre for Drug Design and Discovery (CD3) of the KU Leuven (23, 24). Bacterial strains used in this study are listed in Table 1.

Anti-persister screening assay. The small-molecule library was screened for anti-persister activity as previously described (25). Briefly, stationary phase cells of *P. aeruginosa* cultured in 1:20 TSB were treated for 5 hours with a combination of ofloxacin (10 µg/mL) and test compound (10 µg/mL, corresponding to 20-50 µM of compound). Treated cells were diluted 100-fold into fresh TSB growth medium and incubated in an automated OD plate reader (Bioscreen C, Oy Growth Curves Ab Ltd.) at 37°C with aeration. Previous research revealed a linear relationship between the number of cells incubated in the automated plate reader and the time it took to reach an OD_{600} of 0.6 with y(OD)=-2.1574x(t) + 28.792 and R^2=0.987 (26). Based on this relationship, 105 compounds were selected that showed delayed growth and thus decreased the number of surviving persister cells. Of the 15 most interesting compounds, showing a significant reduction in persister fraction, 5 compounds, belonging to three structurally distinct families, were further confirmed via plate counting. Family 1 proved most

active and 19 chemical analogues of compound SPI001 were subjected to a structure-activity relationship study (Table 2).

MIC assay. Minimal Inhibitory Concentrations (MIC's) were determined using the EUCAST standards for broth microdilution (27). The starting inoculum was adapted to approximately 5 x 10^5 cells/mL and incubated in the presence of a 2-fold dilution series of SPI009 for 24 hours, shaking at 37°C. MIC was determined as the lowest concentration completely inhibiting bacterial growth.

Treatment of isolated persister cells. Persister cells were isolated as described previously, with minor modifications (28). Stationary phase cells were treated for 5 hours with ofloxacin (10 µg/mL). Higher ofloxacin concentrations or increased incubation times did not lead to a further reduction of the number of surviving cells (data not shown). Persisters were washed twice with 0.85% NaCl (5200 x g, 15 minutes, 4 °C) and subsequently used for killing assays, as described below.

Killing assays. Killing assays were performed as previously described (29). Briefly, stationary phase cultures were treated with a combination of antibiotic and compound (anti-persister effect) or compound alone (bactericidal effect). Antibiotic concentrations were chosen to allow only persister cells to survive, indicated by a drop in killing rate. 200 µL volumes of treated culture were dispensed in a 96-well plate and incubated for 5 hours at 37 °C, shaking at 200 rpm. To explore the effect of different treatment regimens, SPI009 was added 0; 5 or 24 hours after the onset of ofloxacin treatment and cells were treated for a total of 72 hours. For time-kill curves different treatment durations were chosen between 15 minutes and 24 hours,

122 comprising treatment with DMSO (0.5%; solvent control), ofloxacin (10 µg/mL), SPI009 alone
123 (17 or 34 µg/mL) and the combination of ofloxacin with SPI009. To assess the effect of different
124 classes of antibiotics stationary phase or exponential phase cultures were treated with 75
125 µg/mL amikacin or 30 µg/mL ceftazidime for 5 hours. After treatment, cells were washed twice
126 in 10 mM $MgSO_4$ (3300 x g, 10 minutes, 4 °C) after which appropriate dilutions were plated on
127 solid growth medium to determine the number of viable cells. Plates were monitored for 72
128 hours to ensure detection of slow growing colonies.

129 **Cytotoxicity assay.** Cytotoxicity was evaluated through colorimetric determination of the lactate
130 dehydrogenase (LDH) activity released from the cytosol of damaged cells (Cytotoxicity Detection
131 Kit PLUS). HEK293-T cells were seeded at concentrations of 1.25 x 10^4 cells/well in 50 µL of
132 appropriate medium and adhesion was allowed overnight. The eukaryotic cells were exposed to
133 increasing concentrations of SPI009 for 24 hours, after which LDH activity was measured
134 according to the manufacturer's guidelines. The relative cytotoxicity was determined as follows:

$$Cytotoxicity\ (\%) = \frac{(Sample\ value - low\ control)}{(High\ control - low\ control)}\ x\ 100$$

135

136 **Hemolytic assay.** Hemolytic activity was assessed as described previously (30), with minor
137 modifications. Defibrinated whole blood from horses (Oxoid) was washed three times with
138 10mM Tris-HCl 0.9% NaCl pH 7.4 (1000 x g, 10 minutes, 4°C) with sequential removing of the
139 buffy coat. Washed erythrocytes were diluted to a final concentration of 2% in 10mM Tris-HCl
140 0.9% HCl pH 7.4 and pre-incubated in 1 mL volumes for 10 minutes (37 °C). 190 µL of
141 erythrocytes were mixed with 10 µL of the different testing agents and incubated at 37°C for 1

hour. 10mM Tris-HCl 0.9% HCl pH 7.4 and 0.1% Triton X-100 were used as negative and positive controls, respectively. Testing concentrations of SPI009 ranged from 8.5 to 34 µg/mL. After incubation the erythrocyte solutions were centrifuged for 5 minutes (3000 x g) and absorbance of the supernatant was measured at 540 nm (Biotek multimode reader) to assess hemolytic damage. Background controls were subtracted from the OD_{540} values and % hemolysis determined relative to the positive control (0.1% Triton X-100). Statistical analysis was performed on control-corrected OD_{540} values using unpaired, one-way ANOVA testing with appropriate correction for multiple comparison (Dunnett) (significance level, $\alpha = 0.05$).

Statistical analysis. Unless mentioned otherwise, all statistical calculations were performed on $\log_{10}$-transformed data using GraphPad Prism software (version 6.01). The effect of different treatments on CFUs was analyzed using unpaired, one-way ANOVA testing, with appropriate correction for multiple comparison (Dunnett) (significance level, $\alpha = 0.05$). Averages are the result of at least three independent repeats.

Results

Identification and structure-activity relationship of anti-persister compound SPI009

A small-molecule library comprising 23 909 diverse molecules (23) was screened to identify compounds that reduce the persister fraction of *P. aeruginosa* in combination with the fluoroquinolone antibiotic ofloxacin (10 µg/mL). To confirm anti-persister activity of the identified compounds, a range of concentrations (0-200 µM) was tested and the efficacy was assessed through viable cell counting. From this analysis, SPI001 was selected for further characterization. To explore the effect of chemical modifications of the molecule on the observed activity, commercially available chemical analogues were purchased and evaluated for their anti-persister effect (Table 2). Based on preliminary experiments (data not shown), the evaluation of the analogues was carried out using a single concentration of 200 µM, corresponding to 68 µg/mL, in combination with 10 µg/mL of ofloxacin. Amongst the analogues that significantly reduced the persister fraction, only SPI009, SPI015 and SPI016 showed an increase in anti-persister activity compared to the original hit SPI001. SPI009, 1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol, had the most pronounced effect, reducing the persister fraction ~7 200-fold ($P < 0.0001$) compared to the treatment with ofloxacin alone. From a structure-activity relationship (SAR) point of view, it is worth noticing that the 7 most active compounds (fold decrease > 39) are all secondary amines (R^3 = hydrogen) whereas most of the other compounds are tertiary amines (except SPI011, SPI014 and SPI020). Moreover, the hydroxyl residue (R^2) does not seem to be essential for good biological activity (see SPI015), although this should be confirmed by the evaluation of additional analogues. Finally, it is striking that the most active analogue (SPI009) is the only compound bearing a phenethyl residue on R^4

177 instead of a benzyl residue, indicating that there is flexibility in the chain length. Based on all
178 these results, SPI009 was selected for further characterization. The antibacterial activity of
179 SPI009 was also evaluated by determination of the MIC value. The MIC value, defined as the
180 minimal concentration to completely inhibit bacterial growth, as calculated for *P. aeruginosa* in
181 1:20 TSB was 150 µM (corresponding to approximately 51 µg/mL).
182
183 **SPI009 directly kills isolated persister cells**
184 To investigate whether SPI009 needs to be administered simultaneously with ofloxacin for
185 maximal activity, the compound was added at different time-points during ofloxacin treatment.
186 For this, *P. aeruginosa* stationary phase cells were first treated with ofloxacin (10 µg/mL) and
187 SPI009 was subsequently added after 0, 5 and 24 hours (Figures 1 a-c). For each experiment,
188 complete eradication of the bacterial population was achieved within 24 hours after adding
189 SPI009. These results indicate that SPI009 can be administrated at any point during treatment
190 without affecting its activity, thus broadening treatment options.
191 Based on these results, we conclude that SPI009 may act in two different ways. Either SPI009
192 wakes up persister cells, hereby rendering them sensitive to the bactericidal action of ofloxacin,
193 or SPI009 kills the persister cells directly. Examples of both strategies have been reported in
194 literature (31–34). To discriminate between these two possibilities, persister cells were isolated
195 and treated with either 10 µg/mL ofloxacin, 17 to 68 µg/mL SPI009 or a combination of
196 ofloxacin and SPI009 (Figure 2). As expected, treatment of the isolated persisters with ofloxacin
197 alone only caused a minor decrease in the number of surviving cells, indicating the effective
198 isolation of persister cells. In contrast, treatment of isolated persister cells with SPI009 caused a

significant decrease in the number of surviving cells ranging between 0.71 ± 0.24 log and complete eradication, as compared to ofloxacin treatment. Combination treatment of SPI009 with ofloxacin was able to completely eliminate all bacterial cells at 34 µg/mL (Figure 2). These results show that SPI009 is capable of directly killing persister cells, even in the absence of antibiotics.

SPI009 is capable of killing both normal and persister cells of *P. aeruginosa*

Previous experiments have clearly shown that SPI009 efficiently targets persister cells. To gain more information about the bactericidal effect on 'normal', non-persister cells, stationary phase cultures were treated with 10 µg/mL ofloxacin or 17 to 68 µg/mL of SPI009. As expected, treatment with 10 µg/mL of ofloxacin causes a significant decrease in the number of surviving cells and allows only persister cells to survive. Treatment of the culture with 34 or 68 µg/mL of SPI009 alone also significantly decreased the number of surviving cells as compared to the control, with reductions of 3.22 ± 0.38 and 6.36 ± 0.39 log units, respectively (Figure 3). Since these decreases are larger than the expected persister fraction, the obtained results clearly show that SPI009 is capable of killing both persister and normal cells.

Killing kinetics of SPI009 as mono- and co-therapy

To assess the killing kinetics of SPI009, stationary phase cultures were exposed to different treatments for 15 minutes to 24 hours. In order to evaluate both the antibacterial and the anti-persister effect of the compound, cells were treated with SPI009 alone (17-34 µg/mL) or with the combination of SPI009 and ofloxacin (10 µg/mL), respectively. The obtained time-kill curves

show a slight biphasic pattern where most killing is obtained in the first 3 to 4 hours (Figure 4). When comparing treatments, the combination treatment of ofloxacin and SPI009 always outcompetes the monotherapies. The combination of ofloxacin with 17 or 34 µg/mL completely eradicates the culture after 24 hours or 5 hours of treatment, respectively. In comparison, 5 hour treatment with 10 µg/mL ofloxacin or 100 µM SPI009 alone causes a 2.87 ± 0.35 log and 4.14 ± 0.35 log decrease in the number of surviving cells, respectively. As suspected, use of SPI009 alone also has an effect on the bacterial culture, causing a maximal 2.36 ± 0.38 and 4.38 ± 0.42 log decrease after 3 hours of treatment with 17 or 34 µg/mL, respectively. The effect of SPI009 is comparable to that of ofloxacin, with a minor increase in bacterial CFUs after 3 hours for SPI009.

Activity of SPI009 is antibiotic-independent

In addition to ofloxacin, other antibiotics used to treat *P. aeruginosa* infections in the clinic include the aminoglycoside amikacin and the cephalosporin ceftazidime (35). As shown in Figure 5a, 5 hour treatment of SPI009 in combination with 75 µg/ml amikacin completely eradicates the stationary phase culture while combination with ofloxacin results in significant 3.36 ± 0.45 and 5.58 ± 0.45 log decreases. The combination treatment of ceftazidime with 34 µg/mL SPI009 also significantly reduced the persister fraction (> 3500 fold) in an exponentially growing culture (Figure 5b). These results clearly show that SPI009 can be combined with different classes of antibiotics and thus possesses antibiotic-independent activity.

SPI009 shows potent activity against several clinical isolates

243 To test whether SPI009 is also active on other clinically relevant strains, we selected five *P.*
244 *aeruginosa* strains to obtain a panel of isolates originating from different human sources, such
245 as burn wounds, urine, throat and bronchus, and with different resistance patterns. Being one
246 of the most dominant bacterial species in CF lungs, several *P. aeruginosa* isolates from the
247 sputum or bronchus of CF patients were included in the panel. Ofloxacin concentrations were
248 optimized for each strain (data not shown). Stationary phase cells of the different cultures were
249 treated with 10 or 100 µg/mL ofloxacin and the combination of ofloxacin with 17 or 34 µg/mL of
250 SPI009. Six out of the eight isolates proved extremely susceptible to killing by the combination
251 treatment, resulting in a (near) complete eradication of the bacterial population (Figure 6). For
252 the least sensitive strains AA249, resistant against aztreonam, ciprofloxacin, ceftazidime,
253 imipenem and meropenem, the combination of ofloxacin with 17 or 34 µg/mL SPI009 still
254 resulted in a significant 2.06 ± 0.55 and 4.69 ± 0.55 log reduction in the number of surviving
255 cells, respectively. Evaluation of CF derived isolates shows modest sensitivity for ofloxacin and
256 demonstrates the potent activity of SPI009 resulting in 4.1 ± 0.3, 4.8 ± 0.3 and 3.7 ± 0.5 log units
257 decrease in surviving cells when treated with the combination of ofloxacin and 34 µg/mL SPI009
258 for DAF87-203, PA1256 and PA1255, respectively. In summary, SPI009 shows potent anti-
259 persister activity in all clinical isolates tested, independent of the source of isolation, and is even
260 capable of strongly reducing survival in multi-drug resistant strains and CF isolates.
261
262 **SPI009 causes no significant cytotoxicity or hemolysis**
263 The *in vitro* activity of SPI009 was clearly demonstrated in different experimental set-ups. An
264 important factor determining its *in vivo* potential is the cytotoxicity of SPI009 in eukaryotic cell

lines. For this, embryonic kidney HEK293-T cells were treated with increasing concentrations of SPI009, ranging from 4.25 to 136 µg/mL for 24 hours. The cytotoxicity was determined by spectroscopic detection of lactate dehydrogenase with three independent repeats resulting in an IC_{50} value of 32.3 ± 0.81 µg/mL. Another important factor considering the clinical use of SPI009 is possible hemolytic activity. This was evaluated by exposing horse erythrocytes to increasing concentrations of SPI009 (8.5 – 34 µg/mL) during 1 hour. Absorbance was measured at 540 nm and percentage hemolysis was determined relative to the positive control (0.1 % Triton X-100). Results indicated no significant difference between the negative control or DMSO (carrier control; 0.5%) and concentrations of up to 34 µg/mL SPI009 or between the different testing agents and 0% hemolysis (Supplementary Figure S1). While the lack of any hemolytic activity is promising, additional adaptation of the chemical structure of SPI009 is desirable to further decrease the moderate cytotoxic effects without affecting its antibacterial properties when applications other than topical treatment of infections are envisioned.

278 **Discussion**

279 The resistance of *P. aeruginosa* against the most commonly used antibiotics is rapidly increasing
280 worldwide and even resistance against colistin and polymyxin B, antibiotics currently used as a
281 last resort in the treatment of *P. aeruginosa* infections, has been reported (4, 36, 37). Besides
282 this rapidly increasing multi-drug resistance, treatment of *P. aeruginosa* infections is further
283 compromised by its ability to form biofilms and the presence of an antibiotic-tolerant persister
284 fraction. Persister cells are able to withstand antibiotic treatment, thereby greatly hampering
285 efficient eradication of the bacterial infection, contributing to the recalcitrant nature of chronic
286 infections and increasing the chance of resistance development (13, 16, 38). Multiple
287 international organizations and research groups acknowledge the increasing threat of bacterial
288 infections, predicted to cause over 10 million deaths annually by 2050 (9), and research into
289 new antibacterial and anti-persister therapies is increasing. In this study, a screen to search for
290 novel anti-persister compounds capable of significantly decreasing the persister fraction in
291 combination with the fluoroquinolone antibiotic ofloxacin led to the identification of 1-((2,4-
292 dichlorophenethyl)amino)-3-phenoxypropan-2-ol (SPI009).

293 Small persister numbers, the different parallel mechanisms behind their formation and
294 conservation and the still limited knowledge greatly impede the rational design of target-based
295 anti-persister therapies. Nevertheless, several anti-persister molecules have been described in
296 literature. Theoretically, there are three approaches to combat bacterial persister cells: (i)
297 waking up persisters and thereby rendering them sensitive to an antibiotic, (ii) directly killing
298 persister cells and (iii) preventing the formation of persister cells. Experiments on both isolated
299 persister cells (Figure 2) and the population level (Figure 3) revealed the ability of SPI009 to

300 significantly decrease the number of surviving cells when added alone. These results clearly
301 demonstrate that SPI009 does not depend on ofloxacin to exert its activity and is capable of
302 directly killing persister cells, categorizing SPI009 in the second class of anti-persister molecules.
303 Other examples of this class include membrane-acting molecules such as peptides shown to
304 target *Escherichia coli* cells, including persisters (39), and NH125, a compound identified
305 through large-scale screening capable of eradicating meticillin-resistant *Staphylococcus aureus*
306 persisters through membrane-permeabilization (40). ADEP4, an acyldepsipeptide, promotes
307 self-digestion in *S. aureus* through constitutive activation of the ClpP protease and combination
308 with rifampicin completely eradicated biofilm infections *in vitro* and *in vivo* (32). More recently,
309 the anti-cancer drugs mitomycin C was described to actively kill persister cells of *E. coli*, *S.*
310 *aureus* and *P. aeruginosa* (41). A more rational approach was used in the development of the
311 Artilysin® Art-175, consisting of a bacteriophage encoded endolysin coupled to a peptide for
312 guidance through the bacterial outer membrane. Art-175 is capable of puncturing the
313 peptidoglycan, resulting in cell lysis, and was shown to be active against persister cells of both *P.*
314 *aeruginosa* (31) and *Acinetobacter baumannii* (42). Another target-based approach led to the
315 identification of a group of quorum-sensing (QS) inhibitors that specifically target the *P.*
316 *aeruginosa* MvfR system. Besides disrupting cell-to-cell communication and decreasing
317 infection, these compounds were the first molecules identified to limit the formation of
318 persister cells in *P. aeruginosa* (43).

319 When treating a normal bacterial population, consisting of both persister and non-
320 persister cells, with SPI0009 it was observed that the activity of the compound is not restricted
321 to non-dividing persister cells but also encompasses normal, non-persister cells (Figure 3). MIC

assays however, revealed a relatively high concentration of 150 µM for SPI009, corresponding to 51 µg/mL, well above the MIC values for most conventional anti-pseudomonal antibiotics (44). Taken together, these results suggest a primary activity against non-dividing or persister cells, with an advantageous secondary effect against normal, actively dividing cells. Coates and co-workers even suggest the use of alternative, more relevant parameters such as the minimal stationary cidal concentrations (MSC) or minimal dormicidal concentration (MDC) for the evaluation of compounds active against non-dividing cells (45–47). Experiments were further focused on stationary phase cultures since the absence of active growth, higher persister fraction and nutritional starvation result in a more pronounced antibiotic tolerance and, partly due to the similarities in biofilms, increased clinical relevance (13, 48). Other anti-persister molecules primarily aimed at targeting non-multiplying cells have been described for *Mycobacterium tuberculosis* (49) and *S. aureus* (50).

While the initial screening was done in combination with ofloxacin, further experiments were undertaken to explore the combination spectrum of SPI009. The limitation of described anti-persister therapies often lies in the fact that they increase the susceptibility of persister cells to one or a limited number of antibiotics. It has however been suggested that the multidrug-tolerant persister population is actually composed of several subpopulations of persister cells, each characterized by their own tolerance profile. This hypothesis is supported by the observed differences in persister fraction upon treatment of a population with distinct classes of antibiotics (51–54). Previous results already indicated an antibiotic-independent effect and additional experiments show that SPI009 is capable of reducing the persister fraction in combination with at least two additional mechanistically different classes of antibiotics

(Figure 5). Combination of SPI009 with the commonly used aminoglycoside amikacin, which acts by inhibiting translation, or ceftazidime, a cephalosporin antibiotic acting on cell wall biogenesis, resulted in complete eradication or significant decreases in the number of surviving cells, proving that SPI009 is capable of targeting multiple subpopulations of persisters. For all antibiotics tested, the addition of SPI009 clearly enhanced bacterial killing, making it a good candidate for the case-specific design of antibacterial co-therapies. Increasing resistance to most antibiotics, together with the current lack of novel anti-pseudomonal compounds has recently increased the use of antibiotic combination therapies. Although the possible negative side-effects need to be studied in more detail, addition of a different antibiotic or non-antibiotic adjuvant to conventional therapy has the potential to lower the concentrations of both agents, reduce treatment times, combine different mode of actions and facilitate the treatment of resistant strains (55–57).

The currently described anti-persister molecules can be classified in three broad categories based on their mechanism of action; (i) compounds that directly kill persister cells, (ii) compounds that sensitize persister cells for antibiotic killing and (iii) compounds that prevent or decrease persister formation. The ability of SPI009 to directly kill persister cells, combined with an excellent activity in combination with distinct classes of antibiotics suggests that SPI009 belongs to this first class. Several compounds capable of directly killing persister cells have been described and include different strategies such as depolarization and destruction of the cell membrane, DNA crosslinking, inhibition of essential enzymes and generation of reactive oxygen species (20, 58, 59). Additional research will be necessary to further unravel the mode of action

365 but preliminary data suggest that SPI009 acts primarily by causing membrane damage (Defraine
366 *et al.*, manuscript in preparation).

367 In conclusion, we identified 1-((2,4-dichlorophenethyl)amino)-3-phenoxypropan-2-ol as a
368 promising compound for the development of future anti-persister therapies. SPI009 is capable
369 of significantly reducing or even eliminating the persister fraction of the Gram-negative
370 pathogen *P. aeruginosa* by direct killing of antibiotic-tolerant persister cells. Due to its activity
371 against both non-dividing and dividing cells and the possibility to combine SPI009 with
372 mechanistically different classes of antibiotics, this molecule has the potential to serve as an
373 adjuvant in antibacterial combination therapies to treat infections containing a mix of bacteria
374 in different growth phases. The absence of any hemolytic effects and the limited cytotoxicity,
375 together with the possibility to further adapt the chemical structure to increase the selectivity
376 index, encourages further research into SPI009 and the development of novel antibacterial
377 strategies in the fight against chronic infections.

378

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391 **References**

392 1. **Folkesson A**, **Jelsbak L**, **Yang L**, **Johansen HK**, **Ciofu O**, **Hoiby N**, **Molin S**. 2012. Adaptation
393 of *Pseudomonas aeruginosa* to the cystic fibrosis airway: an evolutionary perspective. Nat
394 Rev Microbiol **10**:841–851. http://dx.doi.org/10.1038/nrmicro2907.

395 2. **Kerr KG**, **Snelling AM**. 2009. *Pseudomonas aeruginosa*: a formidable and ever-present
396 adversary. J Hosp Infect **73**:338–44. http://dx.doi.org/10.1016/j.jhin.2009.04.020.

397 3. **Pendleton JN**, **Gorman SP**, **Gilmore BF**. 2013. Clinical relevance of the ESKAPE pathogens.
398 Expert Rev Anti Infect Ther **11**:297–308. http://dx.doi.org/10.1586/eri.13.12.

399 4. **Potron A**, **Poirel L**, **Nordmann P**. 2015. Emerging broad-spectrum resistance in
400 *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: Mechanisms and epidemiology.
401 Int J Antimicrob Agents **45**:568–85. http://dx.doi.org/10.1016/j.ijantimicag.2015.03.001.

402 5. **Landman D**, **Bratu S**, **Alam M**, **Quale J**. 2005. Citywide emergence of *Pseudomonas*
403 *aeruginosa* strains with reduced susceptibility to polymyxin B. J Antimicrob Chemother
404 **55**:954–7. http://dx.doi.org/10.1093/jac/dki153.

405 6. **European Centre for Disease Prevention and Control**. 2013. Point prevalence survey of
406 healthcare-associated infections and antimicrobial use in European acute care hospitals
407 2011–2012. ECDC, Stockholm.

408 7. **European Centre for Disease Prevention and Control**. 2015. Annual epidemiological
409 report 2014. Antimicrobial resistance and healthcare-associated infections. ECDC,
410 Stockholm.

411 8. **Tacconelli E**, **Magrini N**. 2017. Global proprity list of antibiotic-resistant bacteria to guide
412 research, discovery, and development of new antibiotics. World Health Organisation.

413 9. **O'Neill J**. 2016. Tackling Drug-Resistant Infections Globally : Final Report and
414 Recommendations. The review on antimicrobial resistance.

415 10. **Mulcahy LR**, **Burns JL**, **Lory S**, **Lewis K**. 2010. Emergence of *Pseudomonas aeruginosa*
416 strains producing high levels of persister cells in patients with cystic fibrosis. J Bacteriol

192:6191–9. http://dx.doi.org/10.1128/JB.01651-09.

11. **Kint CI**, **Verstraeten N**, **Fauvart M**, **Michiels J**. 2012. New-found fundamentals of bacterial persistence. Trends Microbiol **20**:577–585. http://dx.doi.org/10.1016/j.tim.2012.08.009.

12. **Brauner A**, **Fridman O**, **Gefen O**, **Balaban NQ**. 2016. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nat Rev Microbiol **14**:320–30. http://dx.doi.org/10.1038/nrmicro.2016.34.

13. **Fauvart M**, **De Groote VN**, **Michiels J**. 2011. Role of persister cells in chronic infections: clinical relevance and perspectives on anti-persister therapies. J Med Microbiol **60**:699–709. http://dx.doi.org/10.1099/jmm.0.030932-0.

14. **Lafleur MD**, **Qi Q**, **Lewis K**. 2010. Patients with long-term oral carriage harbor high-persister mutants of *Candida albicans*. Antimicrob Agents Chemother **54**:39–44. http://dx.doi.org/10.1128/AAC.00860-09.

15. **Van den Bergh B**, **Michiels JE**, **Fauvart M**, **Michiels J**. 2016. Should we develop screens for multidrug tolerance? Expert Rev Anti Infect Ther **14**:613–616. http://dx.doi.org/10.1080/14787210.2016.1194754.

16. **Levin BR**, **Rozen DE**. 2006. Non-inherited antibiotic resistance. Nat Rev Microbiol **4**:556–62. http://dx.doi.org/10.1038/nrmicro1445.

17. **De Groote VN**, **Fauvart M**, **Kint CI**, **Verstraeten N**, **Jans A**, **Cornelis P**, **Michiels J**. 2011. *Pseudomonas aeruginosa* fosfomycin resistance mechanisms affect non-inherited fluoroquinolone tolerance. J Med Microbiol **60**:329–36. http://dx.doi.org/10.1099/jmm.0.019703-0.

18. **Vogwill T**, **Comfort a. C**, **Furió V**, **MacLean RC**. 2016. Persistence and resistance as complementary bacterial adaptations to antibiotics. J Evol Biol **29**:1223–1233. http://dx.doi.org/10.1111/jeb.12864.

19. **Cohen NR**, **Lobritz M a**, **Collins JJ**. 2013. Microbial persistence and the road to drug resistance. Cell Host Microbe **13**:632–42. http://dx.doi.org/10.1016/j.chom.2013.05.009.

20. **Van Den Bergh B**, **Fauvart M**, **Michiels J**. 2017. Formation, physiology, ecology, evolution and clinical importance of bacterial persisters. FEMS Microbiol Rev 1–33. http://dx.doi.org/10.1093/femsre/fux001.

21. **Harms A**, **Maisonneuve E**, **Gerdes K**. 2016. Mechanisms of bacterial persistence during stress and antibiotic exposure. Science (80-) **354**:aaf4268. http://dx.doi.org/10.1126/science.aaf4268.

22. **Michiels JE**, **Van den Bergh B**, **Verstraeten N**, **Michiels J**. 2016. Molecular mechanisms and clinical implications of bacterial persistence. Drug Resist Updat **29**:76–89. http://dx.doi.org/10.1016/j.drup.2016.10.002.

23. **Liebens V**, **Gerits E**, **Knapen WJ**, **Swings T**, **Beullens S**, **Steenackers HP**, **Robijns S**, **Lippell A**, **O'Neill AJ**, **Veber M**, **Fröhlich M**, **Krona A**, **Lövenklev M**, **Corbau R**, **Marchand A**, **Chaltin P**, **De Brucker K**, **Thevissen K**, **Cammue BP**, **Fauvart M**, **Verstraeten N**, **Michiels J**. 2014. Identification and characterization of an anti-pseudomonal dichlorocarbazol derivative displaying anti-biofilm activity. Bioorg Med Chem Lett **24**:5404–5408. http://dx.doi.org/10.1016/j.bmcl.2014.10.039.

24. **Segers K**, **Klaassen H**, **Economou A**, **Chaltin P**, **Anné J**. 2011. Development of a high-throughput screening assay for the discovery of small-molecule SecA inhibitors. Anal Biochem **413**:90–6. http://dx.doi.org/10.1016/j.ab.2011.02.012.

25. **Liebens V**, **Defraine V**, **Fauvart M**. 2016. A Whole-Cell-Based High-Throughput Screening Method to Identify Molecules Targeting *Pseudomonas aeruginosa* Persister Cells. Methods Mol Biol **1333**:113–120. http://dx.doi.org/10.1007/978-1-4939-2854-5_10.

26. **De Groote VN**, **Verstraeten N**, **Fauvart M**, **Kint CI**, **Verbeeck AM**, **Beullens S**, **Cornelis P**, **Michiels J**. 2009. Novel persistence genes in *Pseudomonas aeruginosa* identified by high-throughput screening. FEMS Microbiol Lett **297**:73–9. http://dx.doi.org/10.1111/j.1574-6968.2009.01657.x.

27. **European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID)**. 2003. Determination

of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution.Clinical Microbiology and Infection **8**:9-15.

28. **Pan J**, **Bahar AA**, **Syed H**, **Ren D**. 2012. Reverting Antibiotic Tolerance of *Pseudomonas aeruginosa* PAO1 Persister Cells by (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one. PLoS One **7**:e45778. http://dx.doi.org/10.1371/journal.pone.0045778.

29. **Liebens V**, **Defraine V**, **Van der Leyden A**, **De Groote VN**, **Fierro C**, **Beullens S**, **Verstraeten N**, **Kint C**, **Jans A**, **Frangipani E**, **Visca P**, **Marchal K**, **Versées W**, **Fauvart M**, **Michiels J**. 2014. A putative de-N-acetylase of the PIG-L superfamily affects fluoroquinolone tolerance in *Pseudomonas aeruginosa*. Pathog Dis **71**:39–54. http://dx.doi.org/10.1111/2049-632X.12174.

30. **Evans BC**, **Nelson CE**, **Yu SS**, **Beavers KR**, **Kim AJ**, **Li H**, **Nelson HM**, **Giorgio TD**, **Duvall CL**. 2013. Ex vivo red blood cell hemolysis assay for the evaluation of pH-responsive endosomolytic agents for cytosolic delivery of biomacromolecular drugs. J Vis Exp e50166. http://dx.doi.org/10.3791/50166.

31. **Briers Y**, **Walmagh M**, **Grymonprez B**, **Biebl M**, **Pirnay J-P**, **Defraine V**, **Michiels J**, **Cenens W**, **Aertsen A**, **Miller S**, **Lavigne R**. 2014. Art-175 Is a Highly Efficient Antibacterial against Multidrug-Resistant Strains and Persisters of Pseudomonas aeruginosa. Antimicrob Agents Chemother **58**:3774–3784. http://dx.doi.org/10.1128/AAC.02668-14.

32. **Conlon BP**, **Nakayasu ES**, **Fleck LE**, **LaFleur MD**, **Isabella VM**, **Coleman K**, **Leonard SN**, **Smith RD**, **Adkins JN**, **Lewis K**. 2013. Activated ClpP kills persisters and eradicates a chronic biofilm infection. Nature **503**:365–370. http://dx.doi.org/10.1038/nature12790.

33. **Pan J**, **Song F**, **Ren D**. 2013. Controlling persister cells of *Pseudomonas aeruginosa* PDO300 by (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one. Bioorg Med Chem Lett **23**:4648–51. http://dx.doi.org/10.1016/j.bmcl.2013.06.011.

34. **Kim J-S**, **Heo P**, **Yang T-J**, **Lee K-S**, **Cho D-H**, **Kim BT**, **Suh J-H**, **Lim H-J**, **Shin D**, **Kim S-K**, **Kweon D-H**. 2011. Selective killing of bacterial persisters by a single chemical compound without affecting normal antibiotic-sensitive cells. Antimicrob Agents Chemother

55:5380–3. http://dx.doi.org/10.1128/AAC.00708-11.

35. **UZ Leuven**. http://www.uzleuven.be/antibioticagids/.

36. **Johansen HK**, **Moskowitz SM**, **Ciofu O**, **Pressler T**, **Høiby N**. 2008. Spread of colistin resistant non-mucoid *Pseudomonas aeruginosa* among chronically infected Danish cystic fibrosis patients. J Cyst Fibros **7**:391–397. http://dx.doi.org/10.1016/j.jcf.2008.02.003.

37. **Lee J-Y**, **Song J-H**, **Ko KS**. 2011. Identification of nonclonal *Pseudomonas aeruginosa* isolates with reduced colistin susceptibility in Korea. Microb Drug Resist **17**:299–304. http://dx.doi.org/10.1089/mdr.2010.0145.

38. **Lewis K**. 2010. Persister cells. Annu Rev Microbiol **64**:357–72. http://dx.doi.org/10.1146/annurev.micro.112408.134306.

39. **Chen X**, **Zhang M**, **Zhou C**, **Kallenbach NR**, **Ren D**. 2011. Control of bacterial persister cells by Trp/Arg-containing antimicrobial peptides. Appl Environ Microbiol **77**:4878–85. http://dx.doi.org/10.1128/AEM.02440-10.

40. **Kim W**, **Conery AL**, **Rajamuthiah R**, **Fuchs BB**, **Ausubel FM**, **Mylonakis E**. 2015. Identification of an Antimicrobial Agent Effective against Methicillin-Resistant *Staphylococcus aureus* Persisters Using a Fluorescence-Based Screening Strategy. PLoS One **10**:e0127640. http://dx.doi.org/10.1371/journal.pone.0127640.

41. **Kwan BW**, **Chowdhury N**, **Wood TK**. 2015. Combatting bacterial infections by killing persister cells with mitomycin C. Environ Microbiol **17**:4406–4414. http://dx.doi.org/10.1111/1462-2920.12873.

42. **Defraine V**, **Scheurmans J**, **Grymonprez B**, **Govers SK**, **Aertsen A**, **Fauvart M**, **Michiels J**, **Lavigne R**, **Briers Y**. 2016. Efficacy of Artilysin® Art-175 against resistant and persistent *Acinetobacter baumannii*. Antimicrob Agents Chemother **60**:AAC.00285-16. http://dx.doi.org/10.1128/AAC.00285-16.

43. **Starkey M**, **Lepine F**, **Maura D**, **Bandyopadhaya A**, **Lesic B**, **He J**, **Kitao T**, **Righi V**, **Milot S**, **Tzika A**, **Rahme L**. 2014. Identification of Anti-virulence Compounds That Disrupt

Quorum-Sensing Regulated Acute and Persistent Pathogenicity. PLoS Pathog **10**:e1004321. http://dx.doi.org/10.1371/journal.ppat.1004321.

44. **EUCAST**. 2017. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 7.1. http://www.eucast.org 0–77. .

45. **Hu Y**, **Shamaei-Tousi A**, **Liu Y**, **Coates A**. 2010. A New Approach for the Discovery of Antibiotics by Targeting Non-Multiplying Bacteria: A Novel Topical Antibiotic for Staphylococcal Infections. PLoS One **5**:e11818. http://dx.doi.org/10.1371/journal.pone.0011818.

46. **Coates A**, **Hu Y**, **Bax R**, **Page C**. 2002. The future challenges facing the development of new antimicrobial drugs. Nat Rev Drug Discov **1**:895–910. http://dx.doi.org/10.1038/nrd940.

47. **Coates ARM**, **Hu Y**. 2008. Targeting non-multiplying organisms as a way to develop novel antimicrobials. Trends Pharmacol Sci **29**:143–150. http://dx.doi.org/10.1016/j.tips.2007.12.001.

48. **Lewis K**. 2007. Persister cells, dormancy and infectious disease. Nat Rev Microbiol **5**:48–56. http://dx.doi.org/10.1038/nrmicro1557.

49. **Bryk R**, **Gold B**, **Venugopal A**, **Singh J**, **Samy R**, **Pupek K**, **Cao H**, **Popescu C**, **Gurney M**, **Hotha S**, **Cherian J**, **Rhee K**, **Ly L**, **Converse PJ**, **Ehrt S**, **Vandal O**, **Jiang X**, **Schneider J**, **Lin G**, **Nathan C**. 2008. Selective killing of nonreplicating mycobacteria. Cell Host Microbe **3**:137–145. http://dx.doi.org/10.1016/j.chom.2008.02.003.

50. **Hu Y**, **Coates A**. 2012. Nonmultiplying Bacteria are Profoundly Tolerant to Antibiotics **211**http://dx.doi.org/10.1007/978-3-642-28951-4.

51. **Spoering AL**, **Lewis K**. 2001. Biofilms and Planktonic Cells of *Pseudomonas aeruginosa* Have Similar Resistance to Killing by Antimicrobials. J Bacteriol **183**:6746–6751. http://dx.doi.org/10.1128/JB.183.23.6746-6751.2001.

52. **Goneau L**, **Yeoh N**. 2014. Selective target inactivation rather than global metabolic dormancy causes antibiotic tolerance in uropathogens. Antimicrob Agents Chemother **58**:2089–97. http://dx.doi.org/10.1128/AAC.02552-13.

53. **Keren I**, **Kaldalu N**, **Spoering A**, **Wang Y**, **Lewis K**. 2004. Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett **230**:13–18. .

54. **Allison KR**, **Brynildsen MP**, **Collins JJ**. 2011. Heterogeneous bacterial persisters and engineering approaches to eliminate them. Curr Opin Microbiol **14**:593–8. http://dx.doi.org/10.1016/j.mib.2011.09.002.

55. **Boyd N**, **Nailor MD**. 2011. Combination antibiotic therapy for empiric and definitive treatment of gram-negative infections: insights from the Society of Infectious Diseases Pharmacists. Pharmacotherapy **31**:1073–84. http://dx.doi.org/10.1592/phco.31.11.1073.

56. **Nakamura I**, **Yamaguchi T**, **Tsukimori A**, **Sato A**, **Fukushima S**, **Matsumoto T**. 2015. New options of antibiotic combination therapy for multidrug-resistant *Pseudomonas aeruginosa*. Eur J Clin Microbiol Infect Dis **34**:83–7. http://dx.doi.org/10.1007/s10096-014-2192-x.

57. **Tamma PD**, **Cosgrove SE**, **Maragakis LL**. 2012. Combination therapy for treatment of infections with gram-negative bacteria. Clin Microbiol Rev **25**:450–70. http://dx.doi.org/10.1128/CMR.05041-11.

58. **Wood TK**. 2016. Combatting bacterial persister cells. Biotechnol Bioeng **113**:476–83. http://dx.doi.org/10.1002/bit.25721.

59. **Helaine S**, **Kugelberg E**. 2014. Bacterial persisters: Formation, eradication, and experimental systems. Trends Microbiol **22**:417–424. http://dx.doi.org/10.1016/j.tim.2014.03.008.

60. **Pirnay J-P**, **Vos D De**, **Mossialos D**, **Vanderkelen A**, **Cornelis P**, **Zizi M**. 2002. Analysis of the *Pseudomonas aeruginosa* oprD gene from clinical and environmental isolates. Environ Microbiol **4**:872–882. http://dx.doi.org/10.1046/j.1462-2920.2002.00281.x.

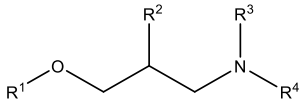
Tables

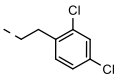
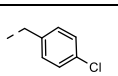
Table 1: Strains used in this study.

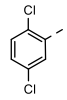
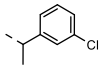
Strain	Description/ Resistance profile	Source or reference
P. aeruginosa PA14	UCBPP-PA14, wild type, Km^R	Pierre Cornelis
P. aeruginosa AA249	Clinical isolate from burn wound; Cip^R, Cf^R, ATM^R, IPM^R, MEM^R	(60)
P. aeruginosa BR642	Clinical isolate from bed pan; ATM^R	(60)
P. aeruginosa BU004	Clinical isolate from throat; ATM^R	(60)
P. aeruginosa BE136	Clinical isolate from bronchus; IPM^R, ATM^R	(60)
P. aeruginosa PA1256	Clinical isolate from CF patient	Françoise van Bambeke (UCL)
P. aeruginosa PA1255	Clinical isolate from CF patient; TIC^R, PIP^R, TZP^R, CAZ^R	Françoise van Bambeke (UCL)
P. aeruginosa PA272	Broncho pulmonary isolate PIP^R, TIC^R	Françoise van Bambeke (UCL)
P. aeruginosa DAF87-203	Sputum isolate from CF patient PIP^R, OFX^R, CST^R	Françoise van Bambeke (UCL)

CIP= ciprofloxacin, CAZ= ceftazidim, ATM= aztreonam, IPM= imipenem, MEM= meropenem, PIP= piperacillin, TIC= ticarcillin, OFX= ofloxacin, CST= colistin, TZP= piperacillin-tazobactam.

591 **Table 2: Structure- activity relationship for SPI009 and chemical analogs.**



Code	R^1	R^2	R^3	R^4	Log (CFU /mL)[1]	Fold decrease compared to ofloxacin	P-value[2]
SPI001		OH	H		1.90	2527.1	<0.05
SPI002		OH			5.53	0.6	NS
SPI003		OH			5.57	0.5	NS
SPI004		OH	H		3.05	176.4	NS
SPI005		OH			4.95	2.2	NS
SPI006		OH			5.53	0.6	NS
SPI007		OH			5.14	1.4	NS
SPI008		OH			4.89	2.6	NS
SPI009		OH	H		1.44	7198.2	<0.0005
SPI010		OH	H		3.71	39.2	<0.005

SPI011		OH	H		4.87	2.7	NS
SPI012		OH			4.76	3.5	NS
SPI013		OH			4.85	2.8	NS
SPI014		OH	H		5.08	1.6	NS
SPI015		H	H		1.74	3647.2	<0.0005
SPI016		OH	H		1.71	3913.6	<0.05
SPI017		OH	CH_3		5.68	0.4	NS
SPI018		OH	H		3.15	142.1	<0.0005
SPI019		OH			5.54	0.6	NS
SPI020		OH	H		5.25	1.1	NS
DMSO 1%					5.30	1.0	

[1] Log (CFU/mL) represents the surviving bacteria after the five hour treatment of a stationary phase culture with the combination of 10 µg/mL ofloxacin and 200 µM compound.

[2] P-value compared to DMSO control treatment. NS: no statistically significant difference (P > 0.05).

595 **Figure legends**

596 **Figure 1: Timing of SPI009 addition to ofloxacin treatment does not influence activity.**
597 Stationary phase cells of a PA14 WT culture were treated for 72 hours with ofloxacin (filled
598 circles) or the combination of ofloxacin with 68 µg/mL SPI009 (filled circles). SPI009 was added
599 to the cultures at a) the beginning of treatment or b) 5 or c) 24 hours after the onset of
600 treatment. During treatment the number of viable cells was determined at 24, 48 and 72 hours
601 by means of CFU counting. Addition of the solvent DMSO (1%, v:v) did not result in any
602 significant killing of the bacterial cells (data not shown). Data points represent the averages of
603 three independent repeats, error bars indicate Standard Error of the Mean (SEM).

604

605 **Figure 2: SPI009 directly kills isolated persister cells.** Persister cells were isolated by means of
606 ofloxacin treatment, after which they were treated for 5 hours with either 1% DMSO, 10 µg/mL
607 ofloxacin (OFX), 17 to 68 µg/mL SPI009 or the combination of ofloxacin with SPI009. After
608 treatment, cells were washed, diluted and plated out to determine the number of surviving
609 persister cells. Data points correspond to the mean of three independent repeats, error bars
610 represent SEM-values. Significant differences are determined on log10-transformed data in
611 comparison to ofloxacin treatment, unless indicated otherwise. * $P \leq 0.05$, ** $P \leq 0.01$, ***$P \leq$
612 0.001, **** $P \leq 0.0001$. ND, not detected.

613

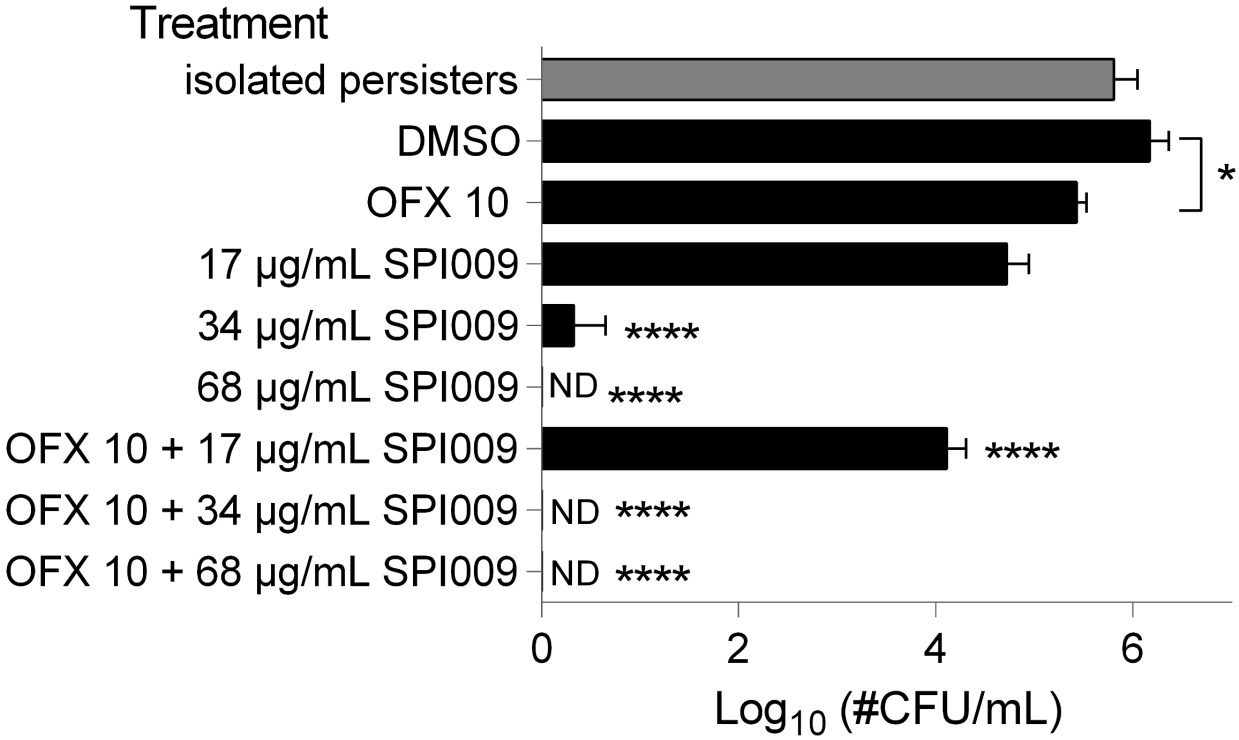
614 **Figure 3: SPI009 targets both normal and persister cells.** Stationary phase cells of *P. aeruginosa*
615 PA14 WT were treated for 5 hours with 1% DMSO (control), 10 µg/mL ofloxacin (OFX) or 17 to
616 68 µg/mL of SPI009. After treatment, cells where washed, diluted and plated onto solid medium

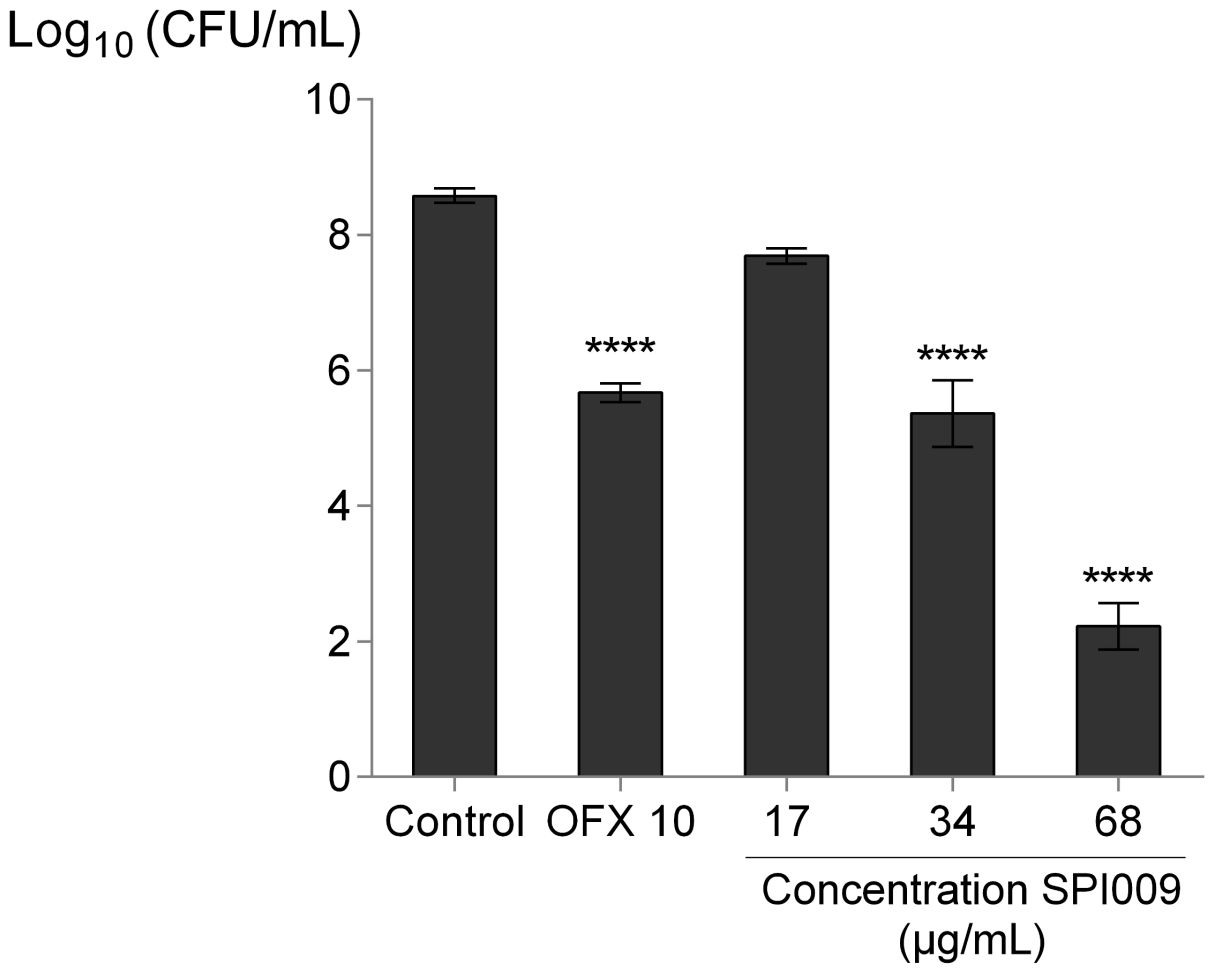
to determine the number of surviving cells. Data points represent the mean of three independent repeats. Error bars represent SEM. Statistical analysis was performed on log-transformed CFU data comparing different treatment conditions with the effect of ofloxacin treatment, where* P ≤ 0.05, ** P ≤ 0.01, ***P ≤ 0.001, **** P ≤ 0.0001. ND, not detected.

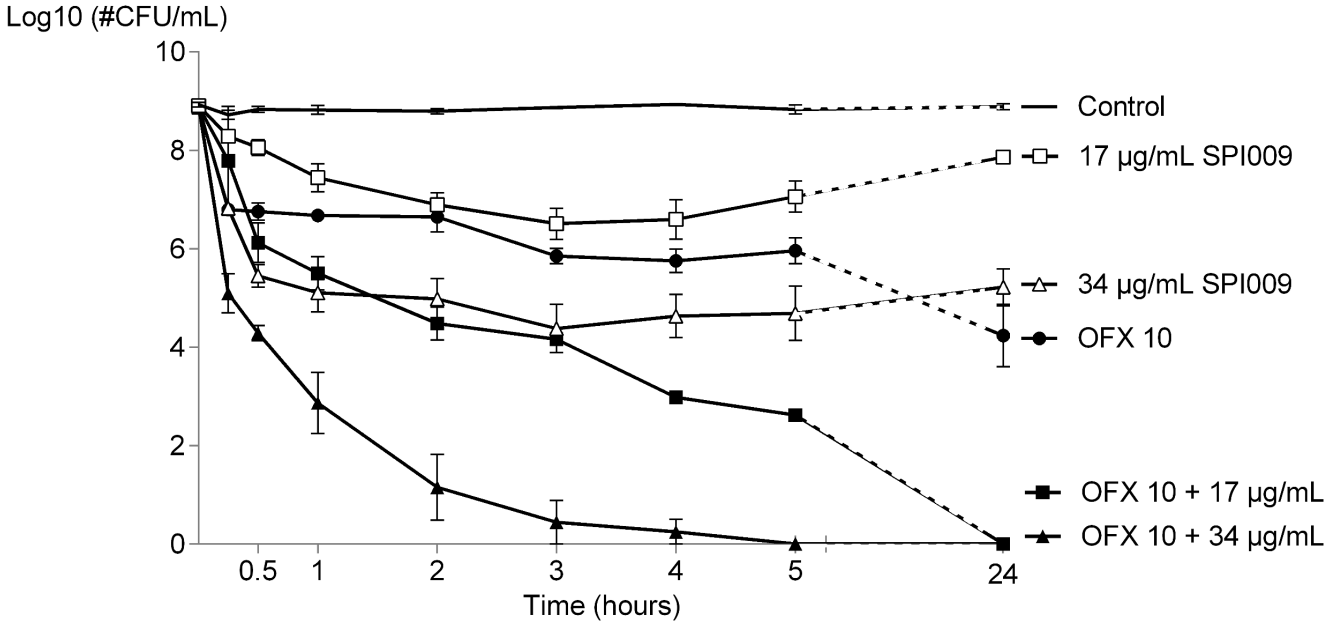
Figure 4: Killing kinetics of SPI009 as a mono- or combination therapy. Stationary phase cells were treated with 1% DSMO (control, solid line); 10 µg/mL ofloxacin (OFX; filled circles); 17 or 34 µg/mL SPI009 (open squares and triangles, respectively) or the combination treatment of ofloxacin with 17 or 34 µg/mL SPI009 (filled squares and triangles, respectively). Samples were taken 15', 30', 1 h, 2 h, 3 h, 4 h, 5 h and 24 hours after the onset of treatment after which they were washed and appropriate dilutions were plated out to assess the number of surviving cells. Data points represent the averages of at least three independent repeats with error bars indicating SEM values.

Figure 5: The combination of SPI009 with antibiotic of distinct classes reveals an antibiotic-independent effect. a) Stationary phase cells and b) exponential phase cells of *P. aeruginosa* PA14 WT were treated for 5 hours with ofloxacin (OFX, 10 µg/mL), amikacin (AMK, 75 µg/ml) or ceftazidime (CAZ, 30 µg/mL), respectively, in combination with 1% DMSO (black bars) or 17-34 µg/mL SPI009 (white bars). Data points correspond to the mean of three independent repeats. Error bars represent SEM. ND, not detected. Statistical analysis compared the effect of antibiotic treatment alone with that of the different combination treatments with significance corresponding to * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001 and **** P ≤ 0.0001. ND, not detected.

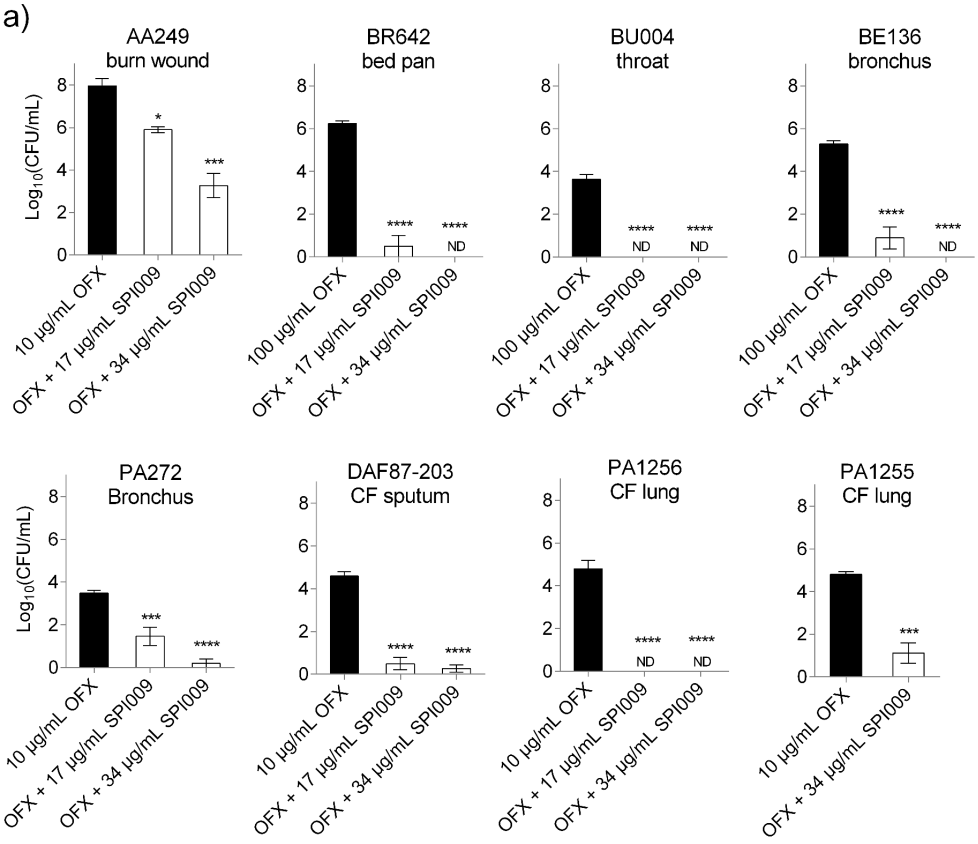
638 **Figure 6: The effect of SPI009 against several clinical *P. aeruginosa* isolates.** a) Stationary phase
639 cells of several clinical isolates were treated for 5 hours with ofloxacin (OFX) alone (black bars)
640 or combined with SPI009 (white bars). After treatment, cells were washed, diluted and plated
641 onto solid medium to determine the number of surviving cells. Data points represent the means
642 of at least three independent repeats, error bars represent SEM. Statistical analysis was done on
643 log10-transformed CFU data with * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. ND,
644 not detected. b) Resistance profiles of the different strains with imipenem (IPM), meropenem
645 (MEM), ceftazidim (CAZ), aztreonam (ATM), ciprofloxacin (CIP), piperacillin (PIP), ticarcillin (TIC),
646 ofloxacin (OFX), colistin (CST), piperacillin-tazobactam (TZP).







a)



b)

Strain	Resistance profile
AA249	IPM, MEM, CAZ, ATM, CIP
BR642	ATM
BU004	ATM
BE136	IPM, ATM
PA272	PIP, TIC
DAF87-203	PIP, OFX, CST
PA1256	/
PA1255	TIC, PIP, TZP, CAZ

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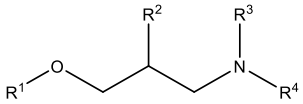
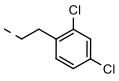
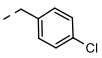
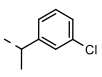
P. aeruginosa BR642	Clinical isolate from bed pan; ATM^R	(60)
P. aeruginosa BU004	Clinical isolate from throat; ATM^R	(60)
P. aeruginosa BE136	Clinical isolate from bronchus; IPM^R, ATM^R	(60)
P. aeruginosa PA1256	Clinical isolate from CF patient	Françoise van Bambeke (UCL)
P. aeruginosa PA1255	Clinical isolate from CF patient; TIC^R, PIP^R, TZP^R, CAZ^R	Françoise van Bambeke (UCL)
P. aeruginosa PA272	Broncho pulmonary isolate PIP^R, TIC^R	Françoise van Bambeke (UCL)
P. aeruginosa DAF87-203	Sputum isolate from CF patient PIP^R, OFX^R, CST^R	Françoise van Bambeke (UCL)

CIP= ciprofloxacin, CAZ= ceftazidim, ATM= aztreonam, IPM= imipenem, MEM= meropenem, PIP= piperacillin, TIC= ticarcillin, OFX= ofloxacin, CST= colistin, TZP= piperacillin-tazobactam.

Table 2: Structure- activity relationship for SPI009 and chemical analogs.



Code	R^1	R^2	R^3	R^4	Log (CFU /mL)[1]	Fold decrease compared to ofloxacin	P-value[2]
SPI001		OH	H		1.90	2527.1	<0.05
SPI002		OH			5.53	0.6	NS
SPI003		OH			5.57	0.5	NS
SPI004		OH	H		3.05	176.4	NS
SPI005		OH			4.95	2.2	NS
SPI006		OH			5.53	0.6	NS
SPI007		OH			5.14	1.4	NS
SPI008		OH			4.89	2.6	NS
SPI009		OH	H		1.44	7198.2	<0.0005
SPI010		OH	H		3.71	39.2	<0.005
SPI011		OH	H		4.87	2.7	NS

Code	R^1	R^2	R^3	R^4	Log (CFU /mL)[1]	Fold decrease compared to ofloxacin	P-value[2]
SPI012		OH			4.76	3.5	NS
SPI013		OH			4.85	2.8	NS
SPI014		OH	H		5.08	1.6	NS
SPI015		H	H		1.74	3647.2	<0.0005
SPI016		OH	H		1.71	3913.6	<0.05
SPI017		OH	CH_3		5.68	0.4	NS
SPI018		OH	H		3.15	142.1	<0.0005
SPI019		OH			5.54	0.6	NS
SPI020		OH	H		5.25	1.1	NS
DMSO 1%					5.30	1.0	

[1] Log (CFU/mL) represents the surviving bacteria after the five hour treatment of a stationary phase culture with the combination of 10 µg/mL ofloxacin and 200 µM compound.

[2] P-value compared to DMSO control treatment. NS: no statistically significant difference (P > 0.05).

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